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# Location-Specific, Unequal Contribution of the N Glycans in Simian Immunodeficiency Virus gp120 to Viral Infectivity and Removal of Multiple Glycans without Disturbing Infectivity

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One of the striking features of human immunodeficiency virus, simian immunodeficiency virus (SIV), and other lentiviruses is extensive N glycosylation of the envelope protein. To assess the requirement of each N glycan for viral infectivity, we individually silenced all 23 N glycosylation sites in the gp120 subunit of SIVmac239 envelope protein by mutagenizing the canonical Asn-Xaa-Thr/Ser N glycosylation motif in an infectious molecular clone, attempted to rescue viruses from the clones, and compared the replication capability of the rescued viruses in MT4 cells. The mutation resulted in either the recovery of a fully infectious virus (category I); recovery of a faster-replicating virus, compared with the parental virus (category II); or no virus recovery (category III). These categorically different sites were not distributed randomly but were clustered. The sites of category I were localized largely in the N-terminal half, whereas the sites of categories II and III were localized in the C-terminal region, including the CD4 binding site, and the central part, including the C loop, respectively. To learn how far SIV can tolerate the removal of glycans, multiplex mutagenesis was also attempted. When they were appreciably distant from one another in the primary sequence, up to five sites could be silenced in combination without disturbing infectivity. On the other hand, it was difficult to silence contiguous sites. Thus, it appeared that a certain degree of sugar chain density over the local region had to be preserved. We discuss the potential utility of these variously deglycosylated mutants for clarifying the role of N glycans in SIV replication in vivo, as well as in the host response, and for designing vaccines and the generation of glycoprotein crystals.

Human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) belong to the genus *Lentivirus* in the family *Retroviridae*. Their envelope glycoproteins consist of the surface and membrane-spanning subunits that are generated by proteolytic cleavage of a precursor polypeptide and are noncovalently associated with each other (for a review, see reference 8). The surface subunits of HIV-1 and SIV, both named gp120, direct target cell recognition and determine viral tropism through interaction with the cell surface receptor CD4 and one of several coreceptors that are members of the chemokine receptor family (for reviews, see references 4, 9, and 30). The transmembrane gp41 (HIV-1) or gp38 (SIV) subunit then promotes fusion of the viral and cellular membranes, releasing the viral contents into the cytoplasm.

There are 26 and 23 potential N-linked glycosylation sites in the gp120s of the HIV-1 strain SF2 and the SIV strain mac239, respectively, whose polypeptide backbones are 482 and 503 amino acid residues long, respectively. There are additionally several O-linked oligosaccharides in HIV-1 (3, 14, 15). The sugar residues thus account for nearly half of the molecular mass of HIV-1 or SIV gp120. Such extensive glycosylation of human and nonhuman lentivirus is very unusual compared with many envelope proteins of different virus families. For instance, the 617-residue-long receptor binding protein H of the measles virus Edmonston strain possesses only five N glycosylation sites, and the 550-residue-long fusion protein F of the same virus has only three.

The HIV-1 gp120 molecules isolated from different sources were found to contain N-linked sugars of the high-mannose type, the complex type, and the hybrid type (11, 21–23). The roles of these sugar chains in the life cycle of HIV-1 have been studied by a variety of approaches, including the use of glycosylation inhibitors and enzymatic deglycosylation. These studies suggested that the three types of N-linked glycans might not have equally important roles in viral syncytium formation and infectivity (reviewed in references 8 and 10). For instance, viral syncytium-inducing ability and infectivity were enhanced by the desialylation of virions or by blocking sugar chain processing to the complex type by a mannosidase I inhibitor (17). The sugar chains should also play different roles, depending on the local amino acid sequence context and on their location on the tertiary glycoprotein structure. Thus, each of the N glycosylation sites was individually silenced for HIV-1 HXB2. However, none of the sites were found to be essential for full infectivity (20). On the other hand, using the same strain, Haggerty et al. (12) found that one of the N glycans in the second constant region (C2) was critically required for infectivity, and Wu et al. (32) found that the two glycans in the second variable region (V2) cooperatively facilitate the proteolytic processing of precursor gp160 to the biologically active gp120/gp41. Furthermore, in the study by Willey et al. (31), the possibility appears

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TABLE 1. Virus rescue and phenotype of rescued virus following mutation of N-linked glycosylation sites in SIVmac239 gp120

Mutation (amino acid position)	Amino acid change	Mutagenic oligonucleotide (5′ to 3′)[a]	Virus rescue and phenotype[b]
37	Asn to Gln	GCTTGGAGGCAAGCGACAATTC	−
39	Thr to Ala	GAGGAATGCGGCAATTCCCC	+ (→)
70	Asn to Gln	GTGGCCCTTCAAGTTACAGAAAG	+ (→)
79	Asn to Gln	GATGCCTGGCAAAATACAGTCAC	+ (→)
114	Asn to Gln	CTATGAGATGCCAAAAAAGTGAGAC	+ (→)
146	Asn to Gln	GTAGACATGGTCCAAGAGACTAGTTC	+ (→)
156	Asn to Gln	GCCCAGGATCAATGCACAGGCTTG	+ (→)
171	Asn to Gln	GCTGTAAATTCCAAATGACAGGG	+ (→)
184	Asn to Gln	GAAAAAAGAGTACCAAGAAACTTGG	+ (→)
198	Asn to Gln	GAACAAGGGCAAAACACTGGTAATG	+ (→)
202	Asn to Gln	AATAACACTGGTCAAGAAAGTAGATG	+ (→)
212	Asn to Gln	CATGAACCACTGTCAAACTTCTGTTATCC	+ (→)
244	Asn to Gln	GCTTAGATGTCAAGACACAAATTAT	+ (→)
247	Asn to Gln	GTAATGACACACAATATTCAGGCTTT	+ (→)
278	Asn to Gln	GGTTTGGCTTTCAAGGAACTAGAG	−
280	Thr to Ala	GCTTTAATGGAGCTAGAGCAG	−
284	Asn to Gln	CTAGAGCAGAACAAAGAACTTATATTT	−
286	Thr to Ala	CAGAAAATAGAGCTTATATTTAC	−
295	Asn to Gln	GGTAGGGATCAAAGGACTATAATTA	−
297	Thr to Ala	GGATAATAGGGCTATAATTAG	−
306	Asn to Gln	AAATAAGTATTATCAACTAACAATGAAA	+ (→)
316	Asn to Gln	GAAGACCAGGACAAAAGACAGTTTTA	−
318	Thr to Ala	CAGGAAATAAGGCAGTTTTACC	−
371	Asn to Gln	GTATACTGGAACTCAAAATACTGATAAA	+ (→)
377	Asn to Gln	CTGATAAAATCCAATTGACGGCTC	−
379	Thr to Ala	AAAATCAATTTGGCGGCTCCTG	+ (→)
460	Asn to Gln	CCTCACGTGTCAATCCACAGTGA	+ (↑)
462	Thr to Ala	GTGTAACTCCGCAGTGACCAG	+ (↑)
476	Asn to Gln	GGATTGATGGACAACAAACTAATATC	+ (→)
479	Asn to Gln	GAAACCAAACTCAAATCACCATGAG	+ (↑)
481	Thr to Ala	CAAACTAATATCGCCATGAGTG	+ (↑)

[a] Underlining indicates mutation sites.
[b] Unsuccessful (−) or successful (+) rescue of virus with similar (→) or increased (↑) replication capability compared with those of the wild type.

to remain open that another N glycan in C2 of the gp120 of strain NL43, similar to HXB2, is critical for infectivity. Thus, the data available are not compatible and do not provide a solid view of the roles of individual glycans in inducing the functional conformation of HIV-1 gp120.

Viral envelope glycoproteins are the major targets of antiviral immune response in infected hosts. In HIV, SIV, and other enveloped viruses, some N glycans tend to shield potential epitopes or hinder the access of antibodies to epitopes (1, 2, 6, 7, 19, 24, 28) while others may be required for inducing antigenically active conformation (13, 29). Again, however, evidence supporting these different roles of N glycans is fragmentary and confined to a few particular glycans within a whole glycoprotein. We are still far from looking at any single whole gp120 molecule and defining which glycans are required for epitope formation, or actually shield potential epitopes, and which polypeptide regions are the masked potential epitopes. We also do not know how our immune system sees those viruses with N glycans thinned out as far as possible. Clarifying these issues would be important not only for understanding HIV pathogenesis but also for designing envelope protein-based vaccines.

The aim of our present study has been to precisely address the requirement for each N glycan in lentivirus infectivity by silencing the glycosylation sites one by one and, further, to learn how far the virus can tolerate removal of multiple glycans. We used SIV, rather than HIV-1, because the goal of this study was to experimentally evaluate the virological and immunological roles of N glycans in vivo. Our results clearly show that the 23 N glycans in gp120 of SIVmac239 are, indeed, not equally important for infectivity but fall into the category of neutral, moderating, or critical glycans with respect to viral infectivity. Moreover, these distinct sites were not distributed randomly but were clustered in particular regions in gp120, suggesting location-specific roles for the N glycans. It was also demonstrated that SIVmac239 could tolerate thinning out at least five N glycans simultaneously.

Each of 23 potential N-linked glycosylation sites in the primary amino acid sequence of SIVmac239 gp120 is denoted by the presence of the canonical N glycosylation motif, Asn-Xaa-Thr/Ser, where Xaa is any amino acid residue except proline. We attempted to rescue viruses from infectious molecular clones in which all 23 N glycosylation sites were individually silenced by changing the first residue, Asn, to a noncanonical Gln residue. The parental clone was the infectious plasmid pBRmac239, into which the proviral sequence of SIVmac239/*nef*-open was cloned (18). A 3,830 bp *Sph*I-*Eco*RI restriction fragment containing the 2,640 nucleotides of the envelope (*env*) gene was excised from pBRmac239 and cloned into the pTZ18U phagemid. Specific nucleotide changes were introduced by using the Muta-gene phagemid in vitro mutagenesis kit version 2 (Bio-Rad, Richmond, Calif.). The oligonucleotide primers used for mutagenesis are listed in Table 1. The mutations introduced were identified by DNA sequencing. The *Sph*I-*Eco*RI fragments containing the introduced mutations were excised from pTZ18U and cloned back into the *Sph*I-*Eco*RI site of pBRmac239. Correct mutations were once again verified by sequencing the final constructs. Any effect or phe-

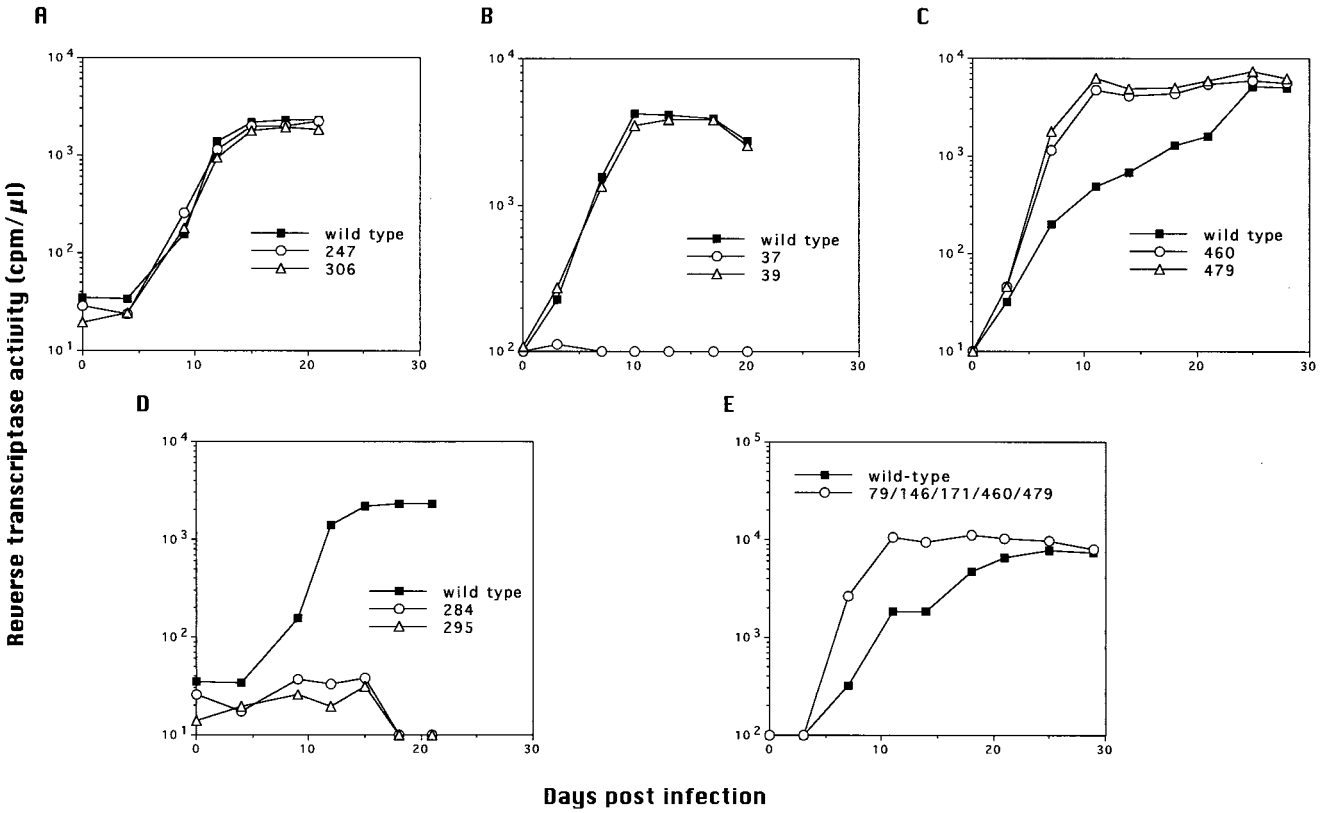

FIG. 1. Representative growth kinetics of N-linked glycosylation site mutants. SW480 cells were transfected with the wild type or N glycosylation mutant plasmids and 2 days later were cocultured with MT4 cells to amplify the recovered virus. The culture supernatants were assayed for reverse transcriptase activities. The start of cocultivation was regarded as day 0. In each set of experiments, the p27 antigen titers on day 0 were comparable among the samples, indicating that the amplification by MT4 cell coculture was initiated with nearly the same amounts of viruses or the noninfectious equivalents. For details see the text.

notype caused by a mutation would not necessarily be a result of removal of the respective N glycans but could result from the amino acid substitution. Thus, whenever the mutation was not neutral with respect to virus rescue and replication, we made an alternative mutagenesis to silence glycosylation by converting the third residue, Thr or Ser, to Ala in the motif (Table 1).

SW480 cells were seeded in a six-well culture plate and 1 day later, at 60 to 80% confluence, were transfected with 3 μg of plasmid DNA by using DOSPER liposomal transfection reagent (Boehringer Mannheim, Mannheim, Germany). Two days later, 5×10^5 MT4 cells were added to each well after the cells were treated with Polybrene (2 mg/ml) for 30 min at 37°C. After 3 days of coculture, the cells were transferred to 25-cm^2 flasks containing 10 ml of RPMI 1640 with 10% fetal calf serum. The culture supernatants of these cells were harvested at 3- to 4-day intervals and assayed for reverse transcriptase activity (16). The culture supernatants were stored at −80°C and used as virus stocks.

The experiments starting with the wild-type cDNA clone were always set aside to make a direct comparison between a mutant and the wild type. In a series of Asn-to-Gln mutagenesis, many sites were found to tolerate the mutation with a fully infectious virus recovery, as exemplified by mutants 247 and 306 (Table 1 and Fig. 1A). These sites, neutral for infectivity, were assigned to category I. Mutant 37 was nonviable, but the alternative mutation, changing the third residue of the N glycosylation motif to create mutant 39, resulted in the production of a fully infectious virus (Fig. 1B). The same was true for sites 377 and 379 (Table 1). These two sites thus fell into category I. Sites 460 and 479, in category II, were unique because mutagenesis of either the first or the third residue resulted in the rescue of viruses capable of replicating significantly faster than the wild type (Fig. 1C and Table 1). The glycans at these positions thus appeared to moderate infectivity. None of the Asn-to-Gln mutations at sites 278, 284, 295, or 316 were able to generate infectious virus (Table 1), as represented by mutants 284 and 295 (Fig. 1D). The same was found by mutagenesis of the respective third residues (Table 1). These sites thus appeared to be essential for either driving correct folding of gp120 or protecting the molecule from proteolysis, and they were assigned to category III.

Removal of a single N glycan from a polypeptide chain should result in a reduction of molecular mass of 2 to 3 kDa. After adjusting the p27 antigen amounts to 200 ng, 5×10^5 MT4 cells were infected with each virus. One week after infection, the cells were metabolically labeled with 100 μCi of EXPRE^{35}S^{35}S[^{35}S] protein-labeling mix (Du Pont-New England Nuclear, Wilmington, Del.) overnight. The cells were then lysed, and envelope proteins in the lysates were immunoprecipitated with serum from a SIVmac239-infected rhesus macaque (*Macaca mulatta*) and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described previously (26). Indeed, all but one, mutant 247, of the recovered mutant viruses displayed a slight mobility shift of their envelope proteins gp160 and gp120 in SDS-PAGE, as represented by mutants 79, 114, 146, 156, 171, 244, 306, 371, and 379 (Fig. 2A). The precursor gp160 molecules migrated as

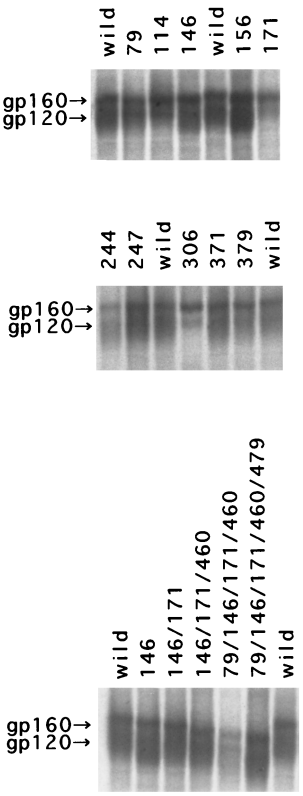

FIG. 2. Electrophoretic mobilities of the envelope proteins encoded by the wild type (wild) and N glycosylation mutants as indicated. Infected MT4 cells were metabolically labeled with EXPRE^{35}S^{35}S[^{35}S] protein-labeling mix overnight. The cells were then lysed, and envelope proteins in the lysates were immunoprecipitated with serum from a SIVmac239-infected rhesus macaque. Sample mobility was analyzed by SDS–7% PAGE. The protein bands corresponding to unprocessed envelope, gp160, and the processed envelope surface component, gp120, are indicated.

a relatively sharp band, because they were in an early phase of glycan addition and could be relatively uniform in size. In contrast, the cleavage products (gp120) were heterogeneous in size, because they had been variously processed. Thus, the mobility shift was more clearly seen for gp160 than for gp120. These results clearly demonstrated that an N glycan was present at each of the sites in the wild-type gp120 and that it was indeed removed by the specific mutation. Whether or not site 247, whose mutagenesis did not result in such a mobility shift as was found for others (Fig. 2A), is actually used remains unclear. However, the glycan addition at this site could likely be sterically blocked by the glycan attached to the preceding site, 244, because the two sites are very close to each other.

The results shown in Table 1 suggested that the sites in distinct categories are not distributed randomly but are clustered in particular parts of gp120. This is more clearly shown when each site is mapped in the linear gp120 sequence with the known structural and functional domains (5) (Fig. 3). The sites in category I were largely distributed in the N-terminal half, while those in categories II and III were distributed in the extreme C terminus, including the CD4 binding domain, and in the middle portion, including the C loop, respectively.

To learn how far SIVmac239 could tolerate the removal of glycans from multiple sites, we attempted to silence as many glycosylation sites as possible. The results of all of our attempts at changing the first residue (Asn to Gln) in the canonical motif are illustrated individually and separately as successful and unsuccessful cases of virus rescue in Fig. 3 (top and bottom, respectively). It was first possible to silence the two sites 146 and 171 simultaneously. Starting with this double mutant, we attempted to additionally silence the intermediate site, 156, or the nearest one, 114 or 184, but no virus was recovered. Thus, it appeared that even though these sites are indispensable as single glycans, some of them absolutely have to be used when others are silenced. In other words, a certain degree of sugar chain density should be preserved over the region either to protect the molecule from proteolysis or to drive its correct folding. Virus rescue was also unsuccessful when we silenced a relatively distant site, 212, or a very distant one, 476, in addition to 146 and 176, suggesting some cooperation between the distant sites or their closeness in the three dimensional structure. On the other hand, it was possible to silence site 460, followed by 79, and finally 479, in addition to 146 and 171 (Fig. 3). Remarkably, this quintuple mutant replicated in cells even faster than the wild type (Fig. 1E). A series of viruses with these single-to-quintuple mutations were analyzed for their gp160 and gp120 mobilities in SDS-PAGE. As shown in Fig. 2B, these glycoprotein molecules migrated faster as the number of mutations increased. This finding clearly verified the removal of N glycans one by one, up to five. It was relatively easy to silence four sites, if the sites were appropriately chosen (Fig. 3).

Our study of single-point mutations, which converted the first or third residue of the canonical Asn-Xaa-Thr/Ser N-linked glycosylation sequences to a noncanonical residue, demonstrated that the 23 N-linked glycosylation sites of SIVmac239 gp120 were not equally important for viral infectivity. They were either neutral, moderating, or critical. A subtle but significant mobility change of mutated gp120 molecules in SDS-PAGE supported the notion that all but one of the neutral and moderating sites are obviously glycosylated in the natural viral life cycle. One of the notable features is that the N glycosylation sites of the three categories are not distributed randomly but tend to cluster in particular regions in the gp120 linear sequence. All four of the sites that were critically required for viral infectivity were mapped to a middle portion, including the C loop. Thus, the cotranslational attachment of sugar chains to these sites, and their processing, appears to be more important than attachment to the other sites for the proper folding and biological functions of gp120. The complex-type N-linked glycans are generally located toward the N termini of membrane-anchored glycoproteins (27). Indeed, in HIV-1 gp120, the central constant region C2 is enriched by high-mannose- and/or hybrid-type chains, while the upstream region including V1 and V2 almost exclusively possesses complex-type chains (21). This suggests that C2 is mostly folded into the interior of the gp120 conformation before its N glycans are further processed to become the complex type, whereas the N-terminal region remains exterior so that the N glycans can be fully processed to the complex type. Monoclonal antibody mapping studies with HIV-1 also suggested that the conserved regions are buried to various extents, while the variable regions are exposed on the surface (reviewed in reference 8). The central region of SIVmac239 gp120 containing the four essential N glycans was well aligned to the region spanning C2 and V3 of HIV-1 gp120, the latter corresponding to the SIV C loop (8). There has been no strong evidence suggesting that the SIV C loop is exposed, as is HIV-1 V3. Furthermore, the sequence variability among strains appears considerably smaller in the SIV C loop than in HIV-1 V3 (5). It is tempting to speculate that the attachment of the four N glycans in the middle portion, including the C loop, is critically required for inducing proper core conforma-

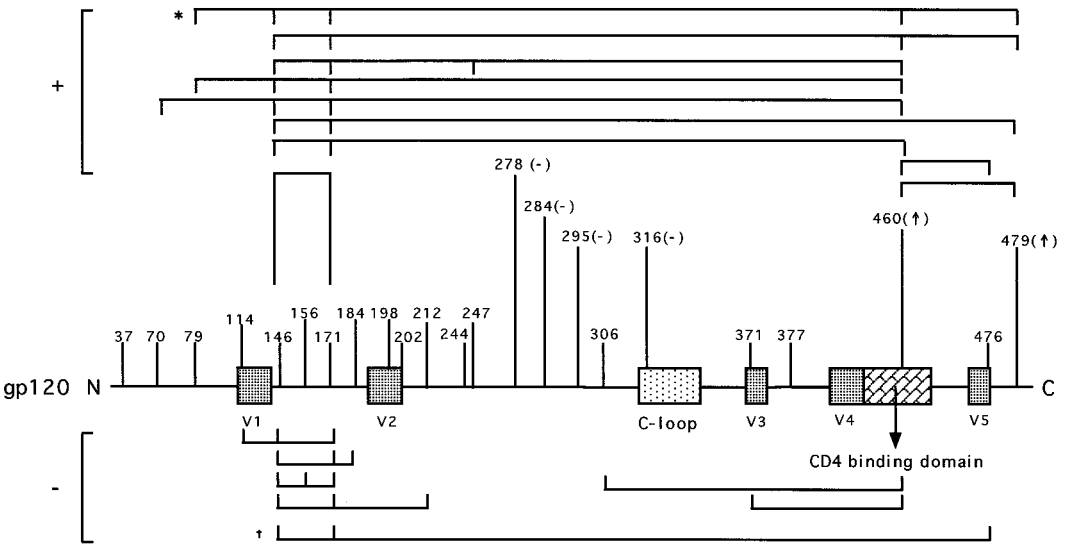

FIG. 3. Summary of the infectivities of 23 N-linked glycosylation site mutants. The numbers indicate the sites of the asparagine residues of the canonical N-linked glycosylation sequence in SIVmac239 gp120. The numbers marked with "(−)" represent the sites whose mutagenesis abolished infectivity, whereas those marked with "(↑)" are the sites whose mutagenesis increased infectivity. Other, unmarked sites are neutral for infectivity, as their mutagenesis did not affect infectivity. Multiplex mutagenesis at two to five sites was also performed. Top (+), combinations resulting in virus generation; bottom (−), combinations that were lethal. For instance, quintuple mutations at sites 79, 146, 171, 460, and 479 (*) allowed the generation of progeny with sufficiently high infectivity whereas triple mutations at 146, 171, and 476 (†) were lethal.

tion of SIVmac239 gp120. The N glycans in C2 of HIV-1 gp120, which appear to be important for infectivity (12, 31), may play a similar role.

On the other hand, both N-terminal and C-terminal N glycosylation sites appear to be less important for proper folding and maturation of SIV gp120. More notable would be that our two single-point mutants, 460 and 479, displayed a faster replication capability, as no analogous cases were reported for HIV-1. Interestingly, these two sites are located within or close to the CD4 binding site. Compared with the wild type, mutant 460 exhibited a higher cell-fusing activity (data not shown) in a gene reporter fusion assay (25). Thus, the N glycans may interfere to some extent with the interaction of gp120 with CD4 and/or with some other early step of infection. However, the conservation of these glycans in natural isolates (5) suggests the advantage of their presence in SIV replication in vivo and persistence in nature. The glycans may mask nearby potential epitopes or sterically inhibit antibody access to the epitopes, thereby helping the virus survive. From the same viewpoint, silencing of up to five sites simultaneously is notable. This type of multiplex mutant will be useful, particularly to depict potential epitopes masked by glycans. Moreover, Doe et al. reported that deglycosylated HIV-1 gp120 primed much more cytotoxic T-lymphocyte response than fully glycosylated gp120 (7). It is thus of considerable interest to learn whether our quintuple-deglycosylation mutant will induce better humoral and cellular responses in rhesus macaques. Its more direct use might be for structural studies, as a high degree of glycosylation is considered to be a major obstacle to the generation of glycoprotein crystals.

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